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(54) Title: HIGH THROUGHPUT ASSAYS FOR THE PROTEOLYTIC ACTIVITIES OF CLOSTRIDIAL NEUROTOXINS

(57) Abstract: In this application is described substrates for high-throughput assays of clostridial neurotoxin proteolytic activities. Two types of substrates are described for use in assays for the proteolytic activities of clostridial neurotoxins: (1) modified peptides or proteins that can serve as FRET substrates and (2) modified peptides or proteins that can serve as immobilized substrates. In both types a fluorescent molecules is present in the substrate, eliminating the requirement for the addition of a fluorigenic reagent. The assays described can be readily adapted for use in automated or robotic systems.

TITLE OF THE INVENTION

High Throughput Assays for the Proteotytic Activities
of Clostridial Neurotoxins

5 .   This application claims priority from U.S. Provisional
application Serial No. 60/235,050 filed on September 25, 2000.

INTRODUCTION

The clostridial neurotoxins consist of tetanus
toxin and the seven immunologically distinct serotypes
10   of botulinum neurotoxin, elaborated by various strains
of *Clostridium tetani* and *Clostridium botulinum*,
respectively. They are among the most potent toxins
known [Simpson, L. L. (1986) Ann. Rev. Pharmacol.
Toxicol . 26: 427-453; Nieman, D. H. (1991) In:
15   Sourcebook of Bacterial Protein Toxins (J. Alouf and
J. Freer, Eds.) pp 303-348, Academic press, New York].
All references cited herein supra and infra are hereby
incorporated in their entirety by reference thereto.
Nonetheless, these toxins have proven to be
20   highly useful tools for research on the mechanisms of
neurotransmitter release [Nieman, D. H. (1991) Trends
Cell Biol. 4: 179-185; Schiavo, et al. (1994) Cell
Biol. 5: 221-229], and are being used as clinical
drugs in humans to treat a rapidly expanding group of
25   muscle dysfunctions including strabismus,
blepharospasm, cervical dystonia, and hemifacial spasm
[Jancovic and Brin (1992) New Engl. J. Med. 324: 1186-
1194; Kessler and Benecke (1997) Neurotoxicology
18(3): 761-770]. Although accidental botulinum
30   intoxication is not considered a major public health
threat, clostridial neurotoxins have long been
recognized as potential biowarfare or bioterrorist
agents [Arnon, S. S., et al. (2001) JAMA 285: 1059-
1070].

2

The clostridial neurotoxins are synthesized by
the bacteria as single-chain proteins of .Mr ~ 150,000,
which are subsequently cleaved by endogenous proteases
to yield a light chain (Mr ~ 50,000) and a heavy chain
5   (Mr ~ 100,000), covalently linked to each other by a
disulfide bond [Bandyopadhyay, et al. (1987) J. Biol.
Chem. 262: 2660-2663]. The heavy chains contain
receptor-binding and translocation domains, required
for entry of neurotoxin into target cells. The light
10   chains are zinc metalloproteases, highly specific for
certain proteins involved in neurotransmitter release
[Montecucco and Schiavo (1994) Mol. Microbiol. 13: 1-
8]. Botulinum serotypes A and E cleave the protein
SNAP-25, while tetanus toxin and botulinum serotypes
15   B, D, F, and G cleave synaptobrevin (also called VAMP)
[Pellizarri, R., et al. (1999) Philos. Trans. Royal
Soc. London B. Biol. Sci. 354: 259-268]. Botulinum
serotype C cleaves both syntaxin and SNAP-25 [Foran,
P., et al. (1996) Biochemistry 35: 2630-2636]. Only
20   one peptide bond is cleaved by each toxin within its
substrate, but this is sufficient to inactivate the
mechanism of neurotransmitter release. Toxicity is
therefore a consequence of clostridial neurotoxin
protease activity.
25   In view of the widespread applications for
clostridial neurotoxins in neurological research and
in medicine, and because of the possibilities for use
as bioweapons, there is an urgent need for highly
sensitive and reproducible assays that can be employed
30   to detect the toxins in potentially contaminated food
or environmental samples, to accurately quantify the
toxins in research reagents or preparations intended
for human clinical use, and in the search for anti-
toxin drugs. Because botulinum neurotoxins are
35   proteases, it follows that practical assays for this

3

activity could form the basis for detection, quantification, and drug-screening systems. However, the development of such assays has been hampered by several factors: (1) As noted above, each botulinum

5   neurotoxin will cleave only one peptide bond in a particular protein, raising the possibility that separate assays would be required for each toxin. (2) A considerable body of evidence has been published which indicates that the substrate recognition

10 requirements of clostridial neurotoxin proteases are unusually large, compared to other proteases, and include discontinuous segments of their respective neuronal target proteins. Therefore, one would anticipate that only intact target proteins or very

15 long polypeptides derived therefrom can function as substrates [for review, see Schiavo, G. et al. (1995) In: Clostridial Neurotoxins (C. Montecucco, Ed.) pp 257-274, Springer-Verlag, Berlin]. (3) The clostridial neurotoxin proteases do not hydrolyze short peptides

20 spanning the cleavage sites, and the tertiary structures of the target proteins are critical elements in substrate recognition [Rossetto O. et al. (1994) Nature 372: 415-416; Schiavo, G. et al. (1995) supra; Washbourne, P. et al. (1997) FEBS Lett. 418: 1-

25 5]. (4) Relatively minor changes in substrate structure, such as the replacement of only one amino acid with a similar one, even at some considerable distance from the cleavage site, can result in complete loss of substrate function [Yamasaki, S. et

30 al. (1994) J. Biol. Chem. 269: 12764-12772; Shone and Roberts (1994) Eur. J. Biochem 225: 263-270; Schmidt and Bostian (1997) J. Prot. Chem. 16: 19-26]. Consequently, introduction of non-natural amino acids and/or bulky aromatic or fluorescent groups would be

35 unlikely to result in a functional substrate.

4

Curently, the most commonly used methods for
detecting botulinum toxins in food and for estimating
concentrations in preparations for clinical use are
the mouse lethality bioassay [Siegel and Metzger
5   (1979) Appl. Environ. Micrbiol. 38: 606-611] and the
antibody neutralization test [Siegel (1988) J. Clin.
Microbiol. 26: 2351-2356]. Both require the use of
animals, can take up to four days to complete, and are
inherently inaccurate. Furthermore, determination of
10   botulinum toxin concentration with the mouse bioassay
cannot be used to predict pharmacological potency
[Pearce, L. B. et al. (1997) Toxicon 35: 1373-1412].
Assays have been published which incorporate
neurotoxin protease activity as one aspect of the
15   overall method [Ekong, T. et al. (1997) Microbiology
143: 3337-3347; Wictome, M. et al. (1999) Appl.
Environ. Microbiol. 65: 3787-3792; Keller, J. et al.
(1999) J. Appl. Toxicol. 19: S13-S17]. Nonetheless,
they are essentially immunoassays, because
20   quantitation of results requires the production and
use of specialized antibodies, capable of
distinguishing between cleaved and uncleaved
substrate, or between cleavage product and intact
substrate. They have been developed only for botulinum
25   serotypes A and B. They require multiple binding,
elution, and washing steps, and are impractical for
true high-throughput systems.
Other assays for the proteolytic activities of
tetanus toxin and of serotypes A and B botulinum
30   toxins have been reported [Shone, C. et al. (1993)
Eur. J. Biochem. 217: 965-971; Cornille, F. et al.
(1994) Eur. J. Biochem. 222: 173-181; Schmidt and
Bostian (1995) J. Prot. Chem. 14: 703-708; Soleilhac,
J.-M. et al. (1996) Anal. Biochem. 241: 120-127].
35   Although these assays could be adapted to high-

5

throughput formats, they include high pressure liquid
chromatography or solid-phase extraction steps, which
add significant time, complexity, and expense to the
procedures.

5   Recently, an assay for the proteolytic activity
of type B botulinum neurotoxin has been published,
which uses a fluoescence resonance energy transfer
(FRET) substrate and does not require physical
separation of products from reactants [Anne, C. et al.
10   (2001) Analyt. Biochem. 291: 253-261]. The publication
describes one substrate, suitable for use with
botulinum serotype B only. Because of the extreme
specificity of each clostridial neurotoxin for a
particular peptide bond in a particular substrate, and
15   the likelihood that structural modifications to any of
the substrates will diminish or abolish cleavability
(see discussion above), nothing may be inferred from
these results with respect to the suitability of
similar modifications to the substrates for the other
20   clostridial neurotoxins, or to modifications of type B
substrate other than those described in the
publication.

        An assay for the proteolytic activity of type A
botulinum toxin has been described (U. S. Patent No.
25   5,965,699) which can be conveniently used to
quantitate, standardize, and compare different
preparations of this toxin. The method is readily
adapted to high-throughput format, to search for
compounds that inhibit botulinum protease activity
30   (i.e. potential anti-botulinum drugs). However, the
assay requires the addition of a fluorigenic reagent
(e.g. fluorescamine, but others are known), which
reacts with one of the proteolytic products to yield a
fluorescent derivative. Furthermore, in some cases,
35   test samples might contain compounds that react

6

directly with the fluorigenic reagent to yield
fluorescent derivatives, interfering with the
measurement of botulinum protease activity.

The assays described in this application are
5   specifically designed to overcome or eliminate all of
the difficulties and drawbacks described above. In
view of the unusually high degree of substrate
specificity and the large substrate recognition
requirements exhibited by all clostridial neurotoxins
10   (v.s.), the extensive substrate modifications required
for the development of the new assays would appear to
render success highly unlikely. Nonetheless, practical
assays have been developed, useful for a wide range of
applications.

15   **SUMMARY**

It is one object of this invention to provide
substrate peptides suitable for use in fluorescence
resonant energy transfer assays (FRET; also known as
quenched-signal assays) for the protease activities of
20   clostridial neurotoxins.

It is another object of the present invention to
provide substrate peptides suitable for use in solid
phase assays for the protease activities of
clostridial neurotoxins.

25   It is another object of this invention to
provide methods for the discovery of compounds that
inhibit or otherwise modulate clostridial neurotoxin
protease activities. Such compounds may be useful in
botulinum toxin clinical applications, or as anti-
30   clostridial neurotoxin drugs.

It is another object of this invention to provide
methods for determining the concentrations of
clostridial neurotoxins in samples (e.g. preparations
of toxin intended for human clinical use) or for
35   detecting the presence of BoNTs in food or

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7

environmental samples, based on the proteolytic
activities of the toxins and utilizing the substrates
described herein.

It is another object of this invention to
5   provide methods for detecting the presence of
clostridial neurotoxins (in food, environmental
samples, etc.), based on the proteolytic activities of
the toxins and utilizing the substrates described
herein.

10   **BRIEF DESCRIPTION OF THE DRAWINGS**

These and other features, aspects, and advantages
of the present invention will become better understood
with regard to the following description, appended
claims, and accompanying drawing.

15   **Figure 1**: Hydrolysis of peptide (1) by
recombinant type A catalytic domain.

Figure 2: Fluorescence solublized by different
concentations of botulinum toxins, serotypes A, B, D,
and F.

20   **DETAILED DESCRIPTION**

The present invention relates to peptides
suitable for the determination of clostridial
neurotoxin proteolytic activities. The term
"clostridial neurotoxins" refers to the seven
25   serotypes of neurotoxin (types A through G, inclusive)
produced by *Clostridium botulinum*, and to tetanus
toxin, produced by *Clostridium tetani*.

The invention includes two types of substrates:
(I), modified peptides or proteins that can serve as
30   FRET or quenched-signal substrates in assays for the
proteolytic activities of clostridial neurotoxins, and
(II), modified peptides or proteins that can serve as
immobilized substrates (i.e. covalently or otherwise
bound to a solid phase) in assays for the proteolytic

8

activities of clostridial neurotoxins. In both types,
a fluorescent molecule is present in the substrate,
eliminating the requirement for the addition of a
fluorigenic reagent. In assays with type (I)

5   substrates, toxin-catalyzed hydrolysis results in a
proportional increase in fluorescence. Therefore,
physical separation of cleavage products from intact
substrate is not necessary. In type (II) assays,
separation of products from intact substrate is

10 accomplished by simply transferring all or part of the
soluble fraction to another container, followed by
quantitation of the fluorescence in the soluble
fraction. Circumstances which favor the use of one
type of assay over the other are discussed below. The

15 assays are called "high-throughput" because lengthy
processing steps such as centrifugation, solid-phase
extraction, or chromatography are not needed.
Therefore, the assays can be readily adapted for use
in automated or robotic systems.

20   Circumstances favoring the use of one type of
assay over the other include: (1), *Cost*. In some
instances, synthesis of a type (I) substrate is more
expensive than a type (II) substrate for the same
serotype. The substrates for botulinum serotype A

25 described in claims (2) and (9) illustrate this
situation. The former, a FRET or type (I) substrate,
is more costly to produce than the latter. Therefore,
if very large numbers of type A assays are
anticipated, economics favors the use of assays

30 incorporating substrate (9), or another type (II)
substrate. (2), *Available instrumentation*. The most
efficient use of type (II) substrates is in multiwell
arrays. However, a fluorometer capable of reading such
arrays is required. If a multiwell fluorometer is not

35 available, use of a FRET substrate would be indicated.

9

(3), *Determinations of clostridial protease kinetic constants*. Initial rates of substrate hydrolyses, catalyzed by clostridial neurotoxin protease activities, are most conveniently determined using

5   FRET or type (I) substrates. Measurements of initial rates are required for calculations of kinetic constants, such as Km, kcat   (turnover number), and the binding affinities of inhibitors or other effectors. (4), *Interference of test samples with*

10   *direct fluorescence measurements*. Properties of certain test compounds, such as quenching, turbidity, or fluorescence, might preclude quantitation of assay results by direct fluorescence measurements. In this situation, use of a solid-phase or type (II) assay is

15   indicated. At the conclusion of the incubation period, samples are removed and the wells are washed to remove all test compounds and enzymes. The amount of uncleaved substrate still bound to each well is then determined by incubation with trypsin, 50-100

20   micrograms per ml, followed by fluorescence measurements. In this situation, the presence of an inhibitor is indicated by a higher fluorescence reading, compared to the control, instead of a lower reading, as in direct assays. (5) *Test samples bound*

25   *to solid matrices*. For example, combinatorial chemistry libraries are often attached to resin beads. In this case, use of a FRET substrate is sometimes more convenient than a solid-phase substrate.
     Type (I) substrates:

30   The following are examples of FRET substrates for the proteolytic activities of clostridial neurotoxins. Each contains a fluorescent group (fluorophore) on one side of the cleavage site, and a molecule that quenches that fluorescence on the other side of the

35   cleavage site. Upon neurotoxin-catalyzed hydrolysis,

the fluorophore and quencher diffuse away from each
other, and the fluorescence signal increases in
proportion to the extent of hydrolysis. Therefore, the
occurrence and rate of hydrolysis may be determined by
5   following the increase in fluorescence with a suitable
fluorimeter. Addition of fluorigenic reagents,
transfer or washing steps, or substrate immobilization
are not required.

Sequences of type A botulinum protease substrates
10   described in U. S. Patent No. 5,965,699, all of which
can be modified as described below for use as FRET
substrates, are herein incorporated by reference.

Substrate (1) (SEQ ID NO:1) is the following peptide:
15

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
S N R T R I D X A N Q R A Z R M L
     Where "X" is N(epsilon)-(2,4-dinitrophenyl)-
lysine and "Z" is S-(fluoresceinyl)-cysteine. This
20   peptide is a substrate for the proteolytic activity of
type A botulinum neurotoxin. Cleavage occurs between
residues 11 (Q) and 12 (R).

Substrate (2) (SEQ ID NO:2) is the following peptide:
25

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
S N R T R I D E A N X R A dcC R M L

     Where "X" is N(epsilon)-(2,4-dinitrophenyl)-
30   lysine and "dcC" is S-(7-dimethylamino-4-methyl-
coumarin-3-carboxamidomethyl)-cysteine. This peptide
is a substrate for the proteolytic activity of type A
botulinum neurotoxin. Cleavage occurs between residues
11 (N(epsilon)-(2,4-dinitrophenyl)-lysine) and 12
35   (R).

Substrate (3)(SEQ ID NO:3) is the following peptide:

```
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
L S E L D D R A D A  L  Q  A  X  A  S  Q  F  E  Z

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
S  A  A  K  L  K  R  K  Y  W  W  K  N  L  K
```

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and "Z" is S-(fluoresceinyl)-cysteine. This peptide is a substrate for the proteolytic activity of type B botulinum neurotoxin. Cleavage occurs between residues 17 (Q) and 18(F).

Substrate (4) (SEQ ID NO:4) is the following peptide:

```
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
L S E L D D R A D A  L  Q  A  G  A  S  X  F  E  dcC

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
S  A  A  K  L  K  R  K  Y  W  W  K  N  L  K
```

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and "dcC" is S-(7-dimethylamino-4-methyl-coumarin-3-carboxamidomethyl)-cysteine. This peptide is a substrate for the proteolytic activity of type B botulinum neurotoxin. Cleavage occurs between residues 17 (N(epsilon)-(2,4-dinitrophenyl)-lysine) and 18(F).

Substrate (5) (SEQ ID NO:5) is the following peptide:

```
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
A Q V D E V V D I M  R  V  N  V  D  K  V  L  X  R
```

12

```
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
D  Q  K  L  Z  E  L  D  D  R  A  D  A  L  Q  A  G  A
```

5   39
    S

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-
lysine and "Z" is S-(fluoresceinyl)-cysteine. This
10 peptide is a substrate for the proteolytic activities
of types D and F botulinum neurotoxins. Type D cleaves
between residues 23 (K) and 24 (L), while type F
cleaves residues 22 (Q) and 23 (K).

15 Substrate (6) (SEQ ID NO:6) is the following peptide:

```
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
A Q V D E V V D I M  R  V  N  V  D  K  V  L  E  R
```

20
```
21 22 23 24 25  26 27 28 29 30 31 32 33 34 35 36 37 38
D  X  K  L  dcC E  L  D  D  R  A  D  A  L  Q  A  G  A
```

    39
    S

25   Where "X" is N(epsilon)-(2,4-dinitrophenyl)-
lysine and "dcC" is S-(7-dimethylamino-4-methyl-
coumarin-3-carboxamidomethyl)-cysteine. This peptide
is a substrate for the proteolytic activities of types
D and F botulinum neurotoxins. Type D cleaves between
30 residues 23 (K) and 24 (L). Cleavage by type F occurs
between residues 22 (N(epsilon)-(2,4-dinitrophenyl)-
lysine) and 23 (K).

Substrate (7) (SEQ ID NO:7) is the following peptide:

```
 1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20
 A  Q  V  D  E  V  V  D  I  M  R  V  N  V  D  K  V  L  E  R

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
 D  X  K  L  mcp E  L  D  D  R  A  D  A  L  Q  A  G  A

39
 S
```

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-
lysine and "mcp" is 2-amino-3-(7-methoxycoumarin-4-
yl)-propionic acid. This peptide is a substrate for
the proteolytic activities of types D and F botulinum
neurotoxins. Type D cleaves between residues 23 (K)
and 24 (L). Cleavage by type F occurs between residues
22 (N(epsilon)-(2,4-dinitrophenyl)-lysine) and 23 (K).

Substrate (8):
        Any peptide or protein that can serve as a
substrate for the proteolytic activity of any
clostridial neurotoxin, said protein or peptide having
been modified to contain a signal moiety on one side
of the cleavage site, and a moiety on the other side
of the cleavage site that quenches or diminishes the
magnitude of that signal. When the substrate is
cleaved by clostridial neurotoxin proteolytic
activity, the two diffuse away from each other and the
signal increases in proportion to the amount of
cleavage that has occurred. Examples of signal and
quench moieties include, respectively: coumarin
derivatives and N(epsilon)-(2,4-dinitrophenyl)-lysine;
coumarin derivatives and nitrotyrosine; fluorescein

14

and rhodamine; fluorescein and N(epsilon)-(2,4-dinitrophenyl)-lysine among others known in the art.

The general concept of FRET assays has been known for many proteases. However, knowledge provided by
5   FRET assays for other proteases cannot be applied directly to the development of FRET substrates for clostridial neurotoxin protease activities, due to the extreme substrate specificities, sensitivities to even minor structural changes in substrates, and the very
10 large substrate recognition requirements of the latter enzymes. In view of these complex and stringent limitations, design of FRET substrates for clostridial neurotoxin protease activities, with respect to types of signal and quench moieties and placement within the
15 substrate sequences, is not obvious.

Type (II) substrates claimed:

Peptides described in substrate (9) - (13) are examples of clostridial neurotoxin substrates,
20 intended for immobilization through reaction of the sulfhydryl groups of the C-terminal cysteine residues in the peptides with maleimide groups, the latter covalently bound to the walls of multiwell plates.

Sequences of type A botulinum protease substrates
25 described in U. S. Patent No. 5,965,699, all of which can be modified as described in this application for use as immobilized substrates, are herein incorporated by reference.

30 Substrate (9) (SEQ ID NO:8) is the following peptide:

1   2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
flG G G S N R T R I D E A N Q R A T R M L

15

```
21 22 23 24
G   G   G   C
```

Where flG is N-fluoresceinyl-glycine. This
peptide is a substrate for the proteolytic activity of
type A botulinum neurotoxin. Cleavage occurs between
residues 14 (Q) and 15 (R).

Substrate (10) (SEQ ID NO:9) is the following
peptide:

```
1  2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
flG G G L S E L D D R A  D  A  L  Q  A  G  A  S  Q
```

```
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
F  E  T  S  A  A  K  L  K  R  K  Y  W  W  K  N  L  K
```

```
39 40 41 42
G  G  G  C
```

Where flG is N-fluoresceinyl-glycine. This
peptide is a substrate for the proteolytic activity of
type B botulinum neurotoxin. Cleavage occurs between
residues 20 (Q) and 21 (F).

Substrate (11) (SEQ ID NO:10) is the following
peptide:

```
1  2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
flG G G A Q V D E V V  D  I  M  R  V  N  V  D  K  V
```

```
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
L  E  R  D  Q  K  L  S  E  L  D  D  R  A  D  A  L  Q
```

16

```
39 40 41 42 43 44 45 46
A  G  A  S  G  G  G  C
```

Where f1G is N-fluoresceinyl-glycine. This
peptide is a substrate for the proteolytic activities
of both types D and type F botulinum neurotoxins. With
type D, cleavage occurs between residues 26 (K) and 27
(L), while type F catalyzes hydrolysis between
residues 25 (Q) and 26(K).

Substrate (12) (SEQ ID NO:11) is the following
peptide:

```
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Z N K L K S S D A Y  K  K  A  W  G  N  N  Q  D  G
```

```
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
V  V  A  S  Q  P  A  R  V  V  D  E  R  E  Q  M  A  I
```

```
39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
S  G  G  F  I  R  R  V  T  N  D  A  R  E  N  E  M  D
```

```
57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
E  N  L  E  Q  V  S  G  I  I  G  N  L  R  H  M  A  L
```

```
75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92
D  M  G  N  E  I  D  T  Q  N  R  Q  I  D  R  I  M  E
```

```
93 94 95 96 97 98 99 100 101 102 103 104 105 106 107
K  A  D  S  N  K  T  R   I   D   E   A   N   Q   R
```

```
108 109 110 111 112 113 114 115 116
A   T   K   M   L   G   S   G   C
```

17

Where Z is S-fluoresceinyl-cysteine. This peptide
is a substrate for the proteolytic activities of types
A and E botulinum neurotoxins. Type A cleaves between
residues 106 (Q) and 107 (R), while type E catalyzes
5 hydrolysis between residues 89 (R) and 90 (I).

Substrate (13) (SEQ ID NO:12) is the following
peptide:

10 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
    Z N K L K S S D A Y K K A W G N N Q D G

    21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
    V V A S Q P A R V V D E R E Q M A I
15

    39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
    S G G F I R R V T N D A R E N E M D

    57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
20 E N L E Q V S G I I G N L R H M A L

    75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92
    D M G N E I D T Q N R Q I D R I M E

25 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107
    K A D S N K T   R   I   D   E   A   N   Q   A

    108 109 110 111 112 113 114 115 116
    A   T   K   M   L   G   S   G   C
30
       Where Z is S-fluoresceinyl-cysteine. This peptide
    is a substrate for the proteolytic activity of type E
    botulinum neurotoxin. Type E catalyzes hydrolysis
    between residues 89 (R) and 90 (I). Replacement of
35 arginine-107 (see sequence in substrate (12)) with

18

alanine prevents cleavage of this substrate by type A
botulinum neurotoxin.

Substrate (14):

5   Any peptide or protein that can serve as a
substrate for the proteolytic activity of any
clostridial neurotoxin, said protein or peptide having
been modified so that it can be attached on one side
of the proteolytic cleavage site to a solid or
10   insoluble material. The attachment point can be on
either side (i.e. C-terminal or N-terminal) of the
cleavage site. Examples of attachment methods include
(but are not limited to): (a), reaction of sulfydryl
groups in the substrate peptide molecules with
15   maleimide groups pre-attached to the solid material
(or, vice versa); (b), binding of biotin groups in
the substrate molecules to avidin or streptavidin
groups on the solid material (or, vice versa).
Examples of solid materials for use in this context
20   include, but are not limited to: (a) multiwell plastic
plates; (b), plastic pins or "dipsticks"; (c), agarose
beads, silica beads, plastic beads, or other types of
spherical or fibrous chromatographic media; (d),
nitrocellulose or other types of sheets or membranes.
25   On the other side of the cleavage site, opposite
from the point of attachment, the substrate contains a
moiety that produces a measurable signal, such as, but
not limited to, a fluorescent group or a radioactive
isotope. When the proteolytic activity of a
30   clostridial neurotoxin cleaves such a substrate, the
product containing the signal is released into
solution. Subsequently, the amount of signal in the
soluble fraction is measured. In addition, if
necessary, the amount of residual bound signal can

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also be measured following solublization of the latter
with a protease such as trypsin.

Immobilized substrate assays have been developed
for many types of enzymes, including other proteases.

5 However, the unusually extensive substrate recognition
requirements of the clostridial neurotoxins and the
relatively large size of the toxin catalytic subunit
(i.e. the light chain, Mr ~50,000) argue against the
attachment of neurotoxin-specific substrates to solid

10 supports. Such an arrangement would be expected to
result in considerable steric hindrance, preventing
free access of the toxin to the substrate on all
sides. Furthermore, the stringent requirements of
clostridial neurotoxins with respect to substrate

15 amino acid sequence indicates that introduction of
bulky fluorescent groups or other signal moieties into
potential substrates would eliminate functionality.
Therefore, application of knowledge gained from
earlier immobilized assays for other enzymes to the

20 development of similar assays for clostridial
neurotoxin protease activities is not encouraged and
is not straightforward.

Abbreviations for the amino acids are:

A   Alanine
25   D   Aspartic acid
E   Glutamic acid
I   Isoleucine
K   Lysine
L   Leucine
30   M   Methionine
N   Asparagine
Q   Glutamine
R   Arginine
S   Serine

T	Threonine
X	N-epsilon-(2,4 dinitrophenyl) lysine
Z	S-(fluoresceinyl)-cysteine
dcC	S-(7-dimethylamino-4-methyl-coumarin-3-carboxamidomethyl) cysteine
mcp	2-amino-3-(7-methoxycoumarin-4-yl)-propionic acid
flG	N-fluoresceinyl-glycine

Peptides can be made with commercially available automated synthesizers, using reagents and protocols obtained from the manufacturers. Amino acids can be obtained in chemically-modified ("protected") forms, designed so that they will react with the free amino group of the preceding residue in the peptide chain, but not with themselves. Upon completion of synthesis, the peptide is cleaved from the resin, protecting groups are removed, and the product is purified. These preparation protocols and others are well within the skill of a person in the art.

In another embodiment, the present invention provides a method for screening compounds which alter BoNT activity, such as inhibitors of BoNT activity or stimulators of BoNT activity. Solutions of BoNT or recombinant botulinum toxin are incubated with each test compound at ambient temperature, transferred to solid supports onto which is immobilized a peptide substrate for the BoNT enzyme being tested as described above, and processed as described above. A toxin incubated with a test compound which exhibits a reduction in the ability of the toxin to cleave the peptide substrate relative to unincubated toxin indicates a inhibitory compound. Alternatively, a toxin incubated with a test compound which exhibits an increase in the ability of the toxin to cleave the

peptide substrate relative to unincubated toxin indicated a stimulatory compound.

In another embodiment, the present invention relates to a kit to search for compounds which inhibit or otherwise alter the protease activities of clostridial neurotoxins. Because the biological effects of clostridial neurotoxins are consequences of their protease activities, it follows that compounds which affect these activities might prove useful as anti-toxin drugs or as tools for further toxin research. Examples of compounds that could be tested include combinatorial sets of chemicals, phage display libraries, or arrays of plant extracts. The kit will contain in close confinement, in a box for example:

-optionally, one or more underivatized multiwell plates ("preincubation plates");

-an equal number of derivatized multiwell substrate plates, which contain substrates for clostridial neurotoxin protease activities bound to the walls of the wells. In most cases, a well will contain substrate for only one clostridial neurotoxin serotype, but combinations of substrates may be used;

-optionally, an equal number of opaque-wall multiwell plates, suitable for use in a multiwell fluorimeter;

-one or more of the clostridial neurotoxins or recombinant light chains thereof;

-optionally dry buffer components.

Solutions of neurotoxin or light chain in buffer are mixed with test compounds in the same buffer in preincubation plate wells and incubated at ambient temperature for approximately 15 - 30 min. This step allows the compounds to exert effects, if any, on the toxins before exposure to the substrates. As controls,

wells containing toxin or light chain without test
compounds are included. Following preincubation, the
solutions are transferred to wells in the derivatized
plates containing immobilized fluorescent substrate
5   specific for the clostridial neurotoxin being tested.
For example, if type A botulinum neurotoxin is tested,
then wells could contain the peptide described in
substrate (9). Substrate plates are incubated for 1 –
3 hours at 30° – 37°C. During this time, the toxin or
10   light chain protease activity will cleave the
immobilized substrate to a certain extent, thereby
solublizing the proteolysis product containing the
fluorescent group. Solutions are then transferred to
the corresponding wells of opaque-wall plates.
15   Fluorescence is then quantitated in a multiwell
fluorimeter. Wells containing compounds that
stimulated or inhibited toxin protease activity will
have more or less fluorescence, respectively, compared
to control wells containing toxin only.
20   In another embodiment, the present invention
relates to a kit for determining the concentrations of
clostridial neurotoxins in samples; for example, it
may be used to monitor the various stages of botulinum
toxin production, intended for human clinical
25   applications. Use of this kit requires knowledge of
which botulinum serotype is present, and the absence
of interfering protease(s). If these conditions are
not met, use of the third kit described below is
indicated. For illustrative purposes, a kit is
30   described for determining concentrations of type A
botulinum neurotoxin. The kit will contain in close
confinement, in a box for example:

        –·FRET substrate for type A botulinum neurotoxin,
described in substrate (2), dry;

23

- optionally, dry buffer components;
- optionally, tween-20 detergent;
- type A botulinum neurotoxin standard.

5   A solution of 30 micromolar substrate is prepared
in water, buffered at pH 7.3 and containing 0.05% v/v
tween-20. Solutions of various known concentrations of
type A botulinum neurotoxin are prepared in the same
buffer. Toxin is mixed with substrate, the increase in
10   fluorescence is measured for a period of time, and the
initial rate of fluorescence increase is determined
from the early (essentially linear) part of the curve.
This is repeated for each known concentration of
toxin, establishing a correlation between toxin
15   concentration and rate. Cleavage rates are then
determined for samples containing unknown
concentrations of type A neurotoxin. By comparison
with the standards, the unknown concentrations may be
calculated.
20   In another embodiment, the present invention
relates to a kit for detecting the presence of
clostridial neurotoxins in samples. In addition to
detection, the kit will also eliminate interfering
proteases that might be present, identify the
25   serotypes of the neurotoxins, and permit calculations
of neurotoxin concentrations. Kits may be used to
screen just a few samples, or large numbers of samples
at once. The kit will contain in close confinement, in
a box for example:
30   - a multiwell plate, containing biotinylated
antibodies against all serotypes of the clostridial
neurotoxins, bound to avidin- or streptavidin- coated
wells. The antibodies are specific for the heavy
chains of the toxins. Generally, a particular well
35   would contain antibodies against only one of the

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clostridial neurotoxins, but all would be represented
on the plate. However, in cases where volume of test
sample is limited, wells could contain more than one
type of antibody. The plate is preferably suitable for
5   use in a multiwell fluorimeter;
       - optionally, dry buffer components for wash buffer;
       - optionally, dry activation buffer components,
       containing buffer, dithiothreitol, and zinc chloride;
       - optionally, Tween-20 detergent;
10   - Clostridial neurotoxin standards.
       - Type (I) FRET substrates and/or type (II)
       substrates, the latter preferably immobilized, for
       example, by attachment to derivatized plastic pins
       which are commercially available. The pins can be used
15   individually, or may be attached to plate lids in an
       array that corresponds to that of the plate wells,
       such that, when a lid is applied to a plate, one pin
       enters each well.
            Solutions of samples suspected of containing
20   clostridial neurotoxins are placed in plate wells.
       Wells containing antibodies against each clostridial
       neurotoxin should receive sample, but if sample volume
       is limited, wells containing multiple anti-neurotoxin
       antibodies would be used. Plates are incubated for
25   approximately one hour at 30° - 37° C. During this
       time, clostridial neurotoxins, if present, will bind
       to the anti-toxin antibodies in the wells. Wells are
       then washed with wash buffer (typically, 50 mM tris,
       0.1% v/v tween-20, pH 7.5, but others may be used) to
30   remove unbound components including, if present,
       proteases other than the clostridial neurotoxins.
       Activation solution (20 mM buffer, 10 mM
       dithiothreitol, 0.50 mM zinc chloride. pH 7.3) is
       added and incubated at 30° - 37° C for 20 - 30 minutes
35   to activate clostridial neurotoxin protease

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activities. Solutions containing FRET substrates are
added, corresponding to the type of neurotoxin that
would be captured by the antibody in a particular
well. After one hour incubation at 30° - 37° C, the
5   amounts of fluorescence in the wells are determined
with a fluorimeter. By comparing fluorescence readings
in test sample wells to those obtained from control
wells (buffers and substrates only) and to readings
from wells containing neurotoxin standards, the
10   presence of a clostridial neurotoxin in a sample may
be detected, and its concentration determined. By
noting the specificity of the antibody in a positive
well, the serotype of the clostridial neurotoxin is
revealed.
15   Alternatively, plastic pins derivatized with the
appropriate type (II) immobilized substrates are
placed in the wells, instead of type (I) FRET
substrates. (Circumstances favoring the use of one
substrate type over the other are discussed in a
20   preceding section). After incubation, the pins are
removed. If the pins were arrayed in a plate lid,
corresponding to the well array, this is accomplished
simply by removing the lid. The amount of fluorescence
in the wells is then determined in a multiwell
25   fluorimeter. Calculations of results are done as
discussed above.
         In situations where wells containing antibodies
against more than one serotype must be used,
differentiation among neurotoxin serotypes is enabled
30   by using substrates containing different fluorophores.
For example, a well might contain antibodies against
botulinum serotypes A, B, E, and F. After incubation
of sample, washing, and activation, a multi-substrate
plastic pin with a combination of four immobilized
35   type (II) substrates is placed in the well. In this

26

example, the pin is derivatized with: (1) substrate
for botulinum type A described in substrate (9); (2)
substrate for botulinum type B described in substrate
(10), modified to replace N-fluoresceinyl-glycine with
5   N-rhodaminyl-glycine as the N-terminal moiety; (3)
substrate for botulinum type E described in substrate
(13), modified to replace S-fluoresceinyl-cysteine
with N-(7-methoxy-coumarin-4-acetyl)-glycine as the N-
terminal moiety; (4) substrate for botulinum type F
10   described in substrate (11), modified to replace N-
fluoresceinyl-glycine with S-(4-acetamido-stilbene-2-
2'-disulfonic acid-4'-carboxamidomethyl)-cysteine.
Because the four fluorophores have distinct and well-
separated emission spectra, the presence of one or
15   more botulinum serotype(s) is/are revealed by
determining the wavelength(s) of any fluorescence
remaining in the well after the removal of the plastic
pin.
        Immobilization of clostridial neurotoxin
20   serotype-specific antibodies may be accomplished in
other ways. For example, instead of substrates, the
antibodies are bound to plastic pins or dipsticks, for
use either individually or in arrays corresponding to
multiwell plates. The pins are immersed in test
25   samples. If clostridial neurotoxins are present, they
will be captured by the antibodies. After rinsing, the
pins are immersed in solutions containing activation
buffer (see above), followed by addition of type (I)
FRET substrates. An increase in fluorescence (compared
30   to appropriate controls) indicates the presence of
neurotoxin. The serotype is revealed by the type of
antibody on the pin. The concentration may be
calculated from a standard curve constructed with
known concentrations of neurotoxin.

27

Alternatively, plastic pins derivatized with the appropriate type (II) immobilized substrates are placed in the wells, instead of type (I) FRET substrates. (Circumstances favoring the use of one
5   substrate type over the other are discussed in a preceding section). After incubation (v.s.), the pins are removed. If the pins were arrayed in a plate lid, corresponding to the well array, this is accomplished simply by removing the lid. The amount of fluorescence
10 in the wells is then determined in a multiwell fluorimeter. Calculations of results are done and conclusions drawn as discussed above.

In another modification, neurotoxin-specific antibodies are bound to the surface of a sheet or
15 membrane in roughly circular spots, corresponding to a multiwell plate array. The sheet or membrane is immersed in the test sample, followed by rinsing. The sheet is then clamped between the upper and lower halves of a multiwell plate, such that the sheet forms
20 the bottom of each well. Alternatively, instead of immersing the sheet in the sample, test samples may be added to the wells, then washed out, or removed by drawing through the sheet by application of a vacuum to the lower plate half. Activation buffer and then
25 type (I) FRET substrates are added to the wells. Results are calculated and conclusions drawn as noted above. Multiwell plates consisting of separable upper and lower halves or chambers, with and without the capability of applying vacuum to the lower chamber,
30 are commercially available and not specifically claimed in this patent application.

The following examples are illustrative of the practice of the invention but should not be read as limiting the scope thereof. It is understood that
35 various modifications could be suggested within the

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spirit and purview of this application and the scope
of the claims.

The following materials and methods were used in
the examples below.

5

Materials and Methods
Enzyme Preparations

Botulinum toxins were obtained from Food Research
Institute, Madison, WI. All preparations appeared to
10 be more than 90% pure, as judged by SDS-PAGE under
reducing conditions. Botulinum toxins were used only
by immune personnel under Biosafety Level-2 controls,
in accordance with the recommendations of the U.S.
Centers for Disease Control and Prevention [*Biosafety*
15 *in Microbiological and Biomedical Laboratories* (1999)
U. S. Government Printing Office, Washington, D. C.].
Maleimide-activated 96-well plates were purchased from
Pierce Chemical Co., Rockford, IL.

Recombinant botulinum type A light chain was
20 expressed in competent *E. coli* BL21 cells (Stratagene,
La Jolla, CA) using a synthetic gene [Ahmed and Smith
(2000) *J. Prot. Chem.* **19**: 475-487] and a pET vector
based upon the T7 RNA polymerase expression system
Studier *et al.* (1990) *Methods Enzymol.* **185**: 60-89].
25 Soluble recombinant type A light chain was purified to
>90% purity by ion exchange chromatography at room
temperature [Li and Singh (1999) *Prot Expr. Purif.* **17**:
339-344]. *12:31 PM*
Peptide Synthesis
30 The peptide synthesizer was a model 431A from
Perkin Elmer-Applied Biosystems, Foster City, CA. We
used protocols, reagents, and chemicals obtained from
the manufacturer. Rink resin was employed to yield a
carboxamide at the C-terminal residues of all
35 peptides. N(alpha)-FMOC-N(epsilon)-2,4-dnitrophenyl-

29

lysine and N-FMOC-2-amino-3(7-methoxycoumarin-4-yl)-
propionic acid were purchased from Bachem Bioscience,
King of Prussia, PA.

Substrates with S-fluoresceinyl cysteine were
5   prepared by reacting the cysteine sulfhydryl group in
the substrate with iodoacetamidofluorescein (Pierce
Chemical Co., Rockford IL). Similarly, substrates
containing S-(7-dimethylamino-4-methyl-coumarin-3-
carboxamidomethyl)-cysteine were prepared by reaction
10   of the cysteine sulfhydryl group with N-(7-
dimethylamino-4-methylcoumarin-3-yl)iodoacetamide
(Molecular Probes, Eugene OR).

Fluorescein was coupled to N-terminal glycine by
deblocking the alpha-amino group, followed by reacting
15   the resin-bound protected peptide with 5-
carboxyfluorescein-N-hydroxysuccinimide ester (Pierce
Chemical Co., Rockford, IL). Alternatively, 5-carboxy-
fluorescein (Aldrich Chemical Co., Milwaukee, WI.) was
loaded into synthesizer cartridges and placed on the
20   instrument as the last residue. Two couplings were
required for complete reaction. After deprotection and
cleavage from the resins, crude peptides were purified
by reverse-phase HPLC.

Preparation of Immobilized-Substrate Assay Plates
25   For covalent coupling to maleimide-activated
multiwell plates, fluorescent peptides were dissolved
at concentrations of 15 to 20 μM in 50 mM Tris, 5 mM
EDTA, 0.1% tween, pH 7.9. All wells received 100 μL
of peptide solution, followed by incubation at ambient
30   temperature for 4 - 5 h. Wells were emptied, then
incubated in succession (200 μL per well, 1 h) with 40
mM 2-mercaptoethanol in Tris-EDTA-tween, then 10 mg/ml
BSA in Tris-tween (no EDTA). Finally, wells were
washed three times with 200 μL of Tris-tween. Assay
35   plates were stored dry at -10° to -20°C.

30

Coupling of Substrates to Derivatized Plastic Pins

      Derivatized pins (also called "gears") can be
obtained from Mimotopes, Raleigh NC., with N-FMOC-
beta-alanine bound through a spacer moiety to the

5   polymer backbone. After removal of the FMOC with
piperidine, the free amino group of the beta-alanine
is iodoacetylated by reaction with iodoacetic
anhydride. Substrate peptide is then covalently bound
by reaction of the sulfhydryl group in the substrate

10   cysteine residue with N-iodoacetyl-beta-alanine on the
pins.

Assays of Botulinum Toxin Proteolytic Activity

      Botulinum toxins (10-20 μg/ml) were preactivated
by incubation at 37°C for 30 min in 20 mM hepes, pH

15   7.3, 10 mM DTT, 0.50 mM $ZnCl_2$, and 0.05% tween.
Assays were conducted in 20 mM hepes, 5 mM DTT, 0.25
mM $ZnCl_2$, and 0.05% tween, with various
concentrations of toxins.

      When recombinant type A light chain was employed

20   in the assays, the lyophilized enzyme was
reconstituted to 35-50 ug/ml with 40 mM hepes/0.05%
tween, pH 7.3, followed by dilution to the desired
concentraion in the same buffer. Unlike whole toxin,
preactivation of light chain is not necessary.

25   Botulinum toxin or recombinant type A light chain were
incubated in the substrate-coated plates for varying
times at 35°C in the dark without agitation.
Appropriate control wells containing buffer only were
included on each plate. Because the maleimide-

30   activated plates have clear walls, they are not
optimized for direct fluorescence measurements.
Therefore, after incubation, aliquots were transferred
to corresponding wells of opaque-wall plates, and
fluorescence was measured with a Wallac 1420 multiwell

35   fluorimeter (PerkinElmer Wallac, Gaithersburg MD,

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USA). Excitation and emission wavelengths were 485
and 535 nm, respectively, with readings of 1 second
per well. All fluorescence values were expressed in
arbitrary units as the mean of triplicate
5   determinations. Error bars are standard deviations.

     Assays employing FRET substrates were done in
cuvettes in a temperature-controlled fluorimeter
(Photon Technology International, Newtown, PA). Toxin
or light chain was mixed with appropriate FRET
10 substrate, placed in the instrument, and fluorescence
was measured for various times.

Example 1

    Operation of the invention using a type (I)
substrate:
15   In this case, the substrate was the peptide
described in substrate (1) above, and the enzyme was a
recombinant preparation of type A botulinum toxin
catalytic domain (also known as "type A light chain").
A solution of 30 micromolar peptide was prepared in
20 water, buffered at pH 7.3, and containing 0.05% v/v
tween-20. Before addition of enzyme, fluorescence was
measured to obtain the background or "zero-time"
fluorescence. Enzyme was then added to a concentration
of two micrograms per ml, and the resulting increase
25 in fluorescence due to proteolysis of the peptide was
measured with time. Assay temperature was 21° C. In
the absence of enzyme, fluorescence changed very
little with time, less than ± 5%. Results are shown in
Fig. 1. The initial rate of hydrolysis can be
30 calculated from the slope of the line in the early
(essentially linear) part of the curve. Using known
concentrations of toxin or light chain, a correlation
between rate and concentration can be established,
allowing calculation of toxin or light chain
35 concentrations in unknown samples.

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Example 2

Operation of the invention using type (II)
substrates:

5   In this example, the substrates were the peptides
described in substrate (9) for botulinum type A,
substrate (10) for type B, and substrate (11) for
types D and F. The "solid material" to which the
substrates were immobilized were 96-well microtiter

10   plates that were chemically modified to contain
maleimide groups (see "Materials and methods"
section). Figure 2 depicts fluorescence solubilized by
different concentrations of botulinum toxins,
serotypes A, B, D, and F (3 hours, 35°C). For each

15   serotype, maximum fluorescence was defined as that
solubilized by 1 microgram per ml toxin under these
conditions. The Y-axis extends above 100% to
accomodate12:31 PM the error bars. Panel (a) shows
BoNT A and B; panel (b) shows BoNT D and F.

20   It is understood that these descriptions,
examples and embodiments are for illustrative purposes
only, and that various modifications would be
suggested within the spirit and purview of this
application and the scope of the appended claims.

25

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What is claimed is:

    1. A clostridial neurotoxin substrate comprising
5   any peptide or protein that can serve as a substrate
    for the proteolytic activity of any clostridial
    neurotoxin, said protein or peptide having been
    modified to contain a signal moiety on one side of the
    cleavage site, and a moiety on the other side of the
10 cleavage site that quenches the magnitude of that
    signal such that when the substrate is cleaved, an
    increase in signal is produced.

    2. The substrate according to claim 1 wherein
15 said clostridial neurotoxin is botulinum neurotoxin
    serotype A.

    3. The substrate according to claim 2 wherein
    said substrate is a peptide identified in SEQ ID NO:1
20 or SEQ ID NO:2.

    4. The substrate according to claim 1 wherein
    said clostridial neurotoxin is botulinum neurotoxin
    serotype B or tetanus toxin.
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    5. The substrate of claim 4 wherein said
    substrate is identified in SEQ ID NO:3 and SEQ ID
    NO:4.

30   6. The substrate according to claim 1 wherein
    said clostridial neurotoxin is botulinum neurotoxin
    serotype D or botulinum neurotoxin serotype F.

    7. The substrate of to claim 6 wherein said
35 substrate is chosen from the group consisting of a

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peptide identified in SEQ ID NO:5, SEQ ID NO:6, and
SEQ ID NO:7.

8. A method for detecting the presence of
5   clostridial neurotoxin proteolytic acitivity in a
sample said method comprising
     mixing the sample with a peptide substrate
according to claim 1, and
     detecting an increase in signal produced from
10   proteolytic cleavage of said substrate.

9. A method for measuring concentration of
neurotoxin in a sample, comprising
     mixing the sample with a peptide substrate
15   according to claim 1,
     measuring an increase in signal with time
produced from proteolytic cleavage of said substrate
and,
     determining the concentration of said neurotoxin
20   by correlation to a standard.

10. The method according to claim 9 wherein said
neurotoxin is botulinum neurotoxin serotype A.

25   11. The method according to claim 10 wherein said
peptide substrate is a peptide identified in SEQ ID
NO:1 or SEQ ID NO:2.

12. The method according to claim 9 wherein said
30   neurotoxin is botulinum neurotoxin serotype B or
tetanus toxin.

13. The method according to claim 12 wherein said
peptide substrate is a peptide identified in SEQ ID
35   NO:3 or SEQ ID NO:4.

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14. The method according to claim 9 wherein said neurotoxin is botulinum neurotoxin serotype D or F.

5   15. The method according to claim 14 wherein said peptide substrate is chosen from the group consisting of a peptide identified in SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7.

10   16. A kit for determining the concentration of a clostridial neurotoxin in a sample, the kit containing in close confinement,
        (i) one or more peptide substrates according to claim 1 cleavable by said clostridial neurotoxin;
15   (ii) said clostridial neurotoxin standard.

17. The kit according to claim 16 wherein said clostridial neurotoxin is botulinum neurotoxin serotype A and the peptide substrate is one or both of
20   the peptides identified in SEQ ID NO:1 and SEQ ID NO:2.

18. The kit according to claim 16 wherein said clostridial neurotoxin is botulinum neurotoxin
25   serotype B or tetanus toxin and the peptide substrate is one or both of the peptides identified in SEQ ID NO:3 and SEQ ID NO:4.

19. The kit according to claim 16 wherein said
30   clostridial neurotoxin is botulinum neurotoxin serotype D or F and the peptide substrate is one or more of the peptides identified in SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7.

20. A botulinum neurotoxin substrate comprising
any peptide or protein that can serve as a substrate
for the proteolytic activity of any clostridial
neurotoxin, said protein or peptide having been
5   modified so that it can be attached on one side of the
proteolytic cleavage site to a solid material.

21. The substrate according to claim 20 wherein
said clostridial neurotoxin is botulinum neurotoxin
10 serotype A.

22. The substrate according to claim 21 wherein
said substrate is a peptide identified in SEQ ID NO:8
or SEQ ID NO:11.
15

23. The substrate according to claim 20 wherein
said clostridial neurotoxin is botulinum neurotoxin
serotype B or tetanus toxin.

20   24. The substrate of claim 23 wherein said
substrate is identified in SEQ ID NO:9.

25. The substrate according to claim 20 wherein
said clostridial neurotoxin is botulinum neurotoxin
25 serotype D or serotype F.

26. The substrate of claim 25 wherein said
substrate is a peptide identified in SEQ ID NO:10.

30   27. The substrate of claim 20 wherein said
botulinum neurotoxin is botulinum neurotoxin serotype
E.

37

28. The substrate of claim 27 wherein said substrate is a peptide identified in SEQ ID NO:11 and 12.

5   29. A method for detecting the presence of clostridial neurotoxin proteolytic acitivity in a sample said method comprising

mixing the sample with a peptide substrate according to claim 20, and

10   detecting an increase in signal produced from proteolytic cleavage of said substrate.

30. A method for measuring concentration of neurotoxin in a sample, comprising

15   mixing the sample with a peptide substrate according to claim 20,

measuring an increase in signal with time produced from proteolytic cleavage of said substrate and,

20   determining the concentration of said neurotoxin by correlation to a standard.

31. The method according to claim 30 wherein said neurotoxin is botulinum neurotoxin serotype A.

25

32. The method according to claim 31 wherein said peptide substrate is a peptide identified in SEQ ID NO:8 or SEQ ID NO:11.

30   33. The method according to claim 30 wherein said neurotoxin is botulinum neurotoxin serotype B or tetanus toxin.

38

34. The method according to claim 33 wherein said peptide substrate is a peptide identified in SEQ ID NO:9.

5   35. The method according to claim 30 wherein said neurotoxin is botulinum neurotoxin serotype D or F.

36. The method according to claim 35 wherein said peptide substrate is a peptide identified in SEQ ID
10   NO:10.

37. The method according to claim 30 wherein said neurotoxin is botulinum neurotoxin serotype E.

15   38. The method according to claim 37 wherein said peptide substrate is a peptide identified in SEQ ID NO:11 or SEQ ID NO:12.

39. A kit for determining the concentration of a
20   clostridial neurotoxin in a sample, the kit containing in close confinement,
        (i) one or more peptide substrates according to claim 20 cleavable by said clostridial neurotoxin;
        (ii) said clostridial neurotoxin standard.
25

40. The kit according to claim 39 wherein said clostridial neurotoxin is botulinum neurotoxin serotype A and the peptide substrate is one or both of the peptides identified in SEQ ID NO:8 and SEQ ID
30   NO:11.

41. The kit according to claim 39 wherein said clostridial neurotoxin is botulinum neurotoxin serotype B or tetanus toxin and the peptide substrate
35   is a peptide identified in SEQ ID NO:9.

42. The kit according to claim 39 wherein said
clostridial neurotoxin is botulinum neurotoxin
serotype D or F and the peptide substrate is a peptide
5   identified in SEQ ID NO:10.

43. The kit according to claim 39 wherein said
clostridial neurotoxin is botulinum neurotoxin
serotype E and the peptide substrate is one or more of
10   the peptides identified in SEQ ID NO:11 or SEQ ID
NO:12.

44. A method for identifying inhibitors or
enhancers of proteolytic activity of a clostridial
15   neurotoxin comprising:
        preincubating a neurotoxin with a test compound
to make a neurotoxin-compound solution,
        exposing said solution to a substrate of said
neurotoxin according to claim 20,
20   measuring signal resulting from the proteolysis
of said substrate by said neurotoxin, and
        comparing said signal with controls,
        wherein an increase in signal indicates a
compound which enhances neurotoxin activity and a
25   decrease in signal indicates a compound with inhibits
said neurotoxin.

45. The method according to claim 44 wherein said
neurotoxin is botulinum neurotoxin serotype A and the
30   substrate is a peptide identified in SEQ ID NO:8 or
SEQ ID NO:11.

46. The method according to claim 44 wherein said
neurotoxin is botulinum neurotoxin serotype B or

tetanus toxin and the substrate is a peptide
identified in SEQ ID NO:9.

47. The method according to claim 44 wherein said
5   neurotoxin is botulinum neurotoxin serotype D or F and
the substrate is a peptide identified in SEQ ID NO:10.

48. The method according to claim 44 wherein said
neurotoxin is botulinum neurotoxin serotype E and the
10   substrate is one or more of the peptides identified in
SEQ ID NO:11 or SEQ ID NO:12.

49. A method for identifying a serotype of a
clostridial neurotoxin in a sample suspected of
15   containing a neurotoxin, the method comprising
      incubating the sample with antibodies against
each clostridial neurotoxin such that a neurotoxin is
bound to its serotype-specific antibody,
      removing unbound components,
20   adding activation solution such that clostridial
protease is activated,
      adding solutions containing clostridial
neurotoxin peptide substrates according to claim 1 to
said activated protease,
25   detecting signal generated from proteolysis of
said substrate by said protease, wherein a signal
above control indicates presence of a neurotoxin, and
      determining the serotype of the clostridial
neurotoxin by noting the specificity of the antibody.
30

50. The method of claim 49 wherein the antibodies
are bound to a solid material.

51. The method of claim 50 wherein the solid
35   material is a multiwell plate.

41

52. The method of claim 51 wherein each well contains an antibody specific for a different neurotoxin serotype.

5

53. The method of claim 49 wherein each peptide substrate is labeled with a different signal.

54. A kit for identifying a serotype of a

10   clostridial neurotoxin in a sample suspected of containing a neurotoxin, comprising

serotype-specific antibodies
clostridial neurotoxin standards, and
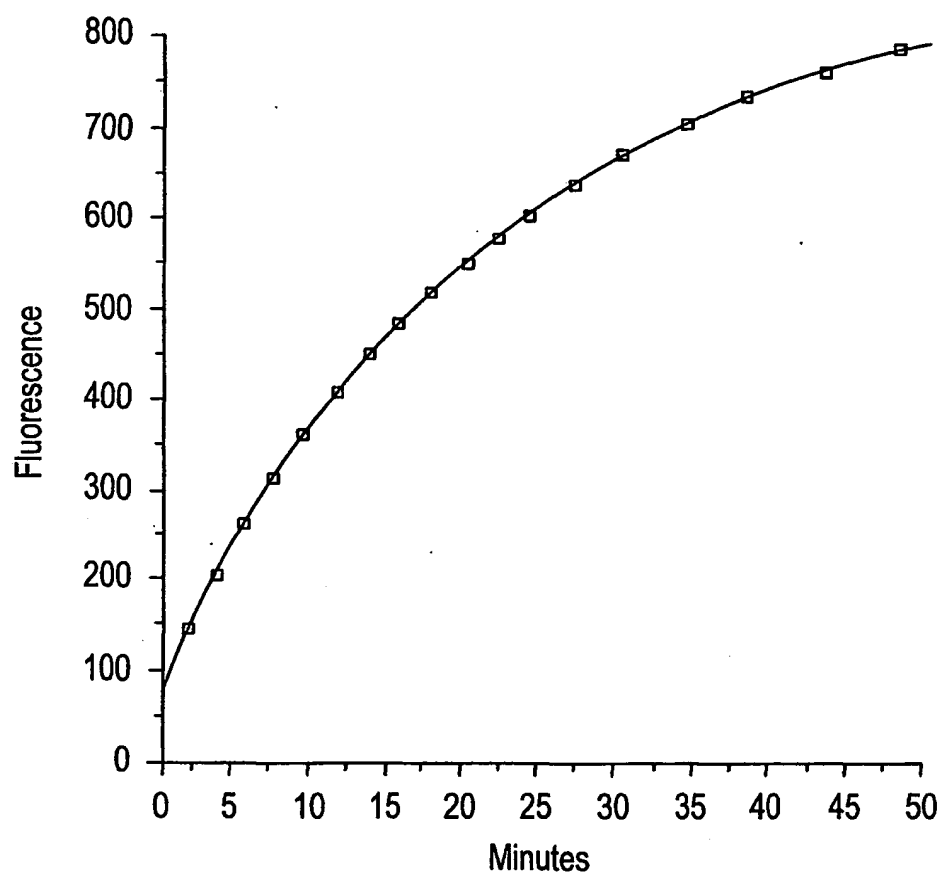peptide substrates according to claim 1.
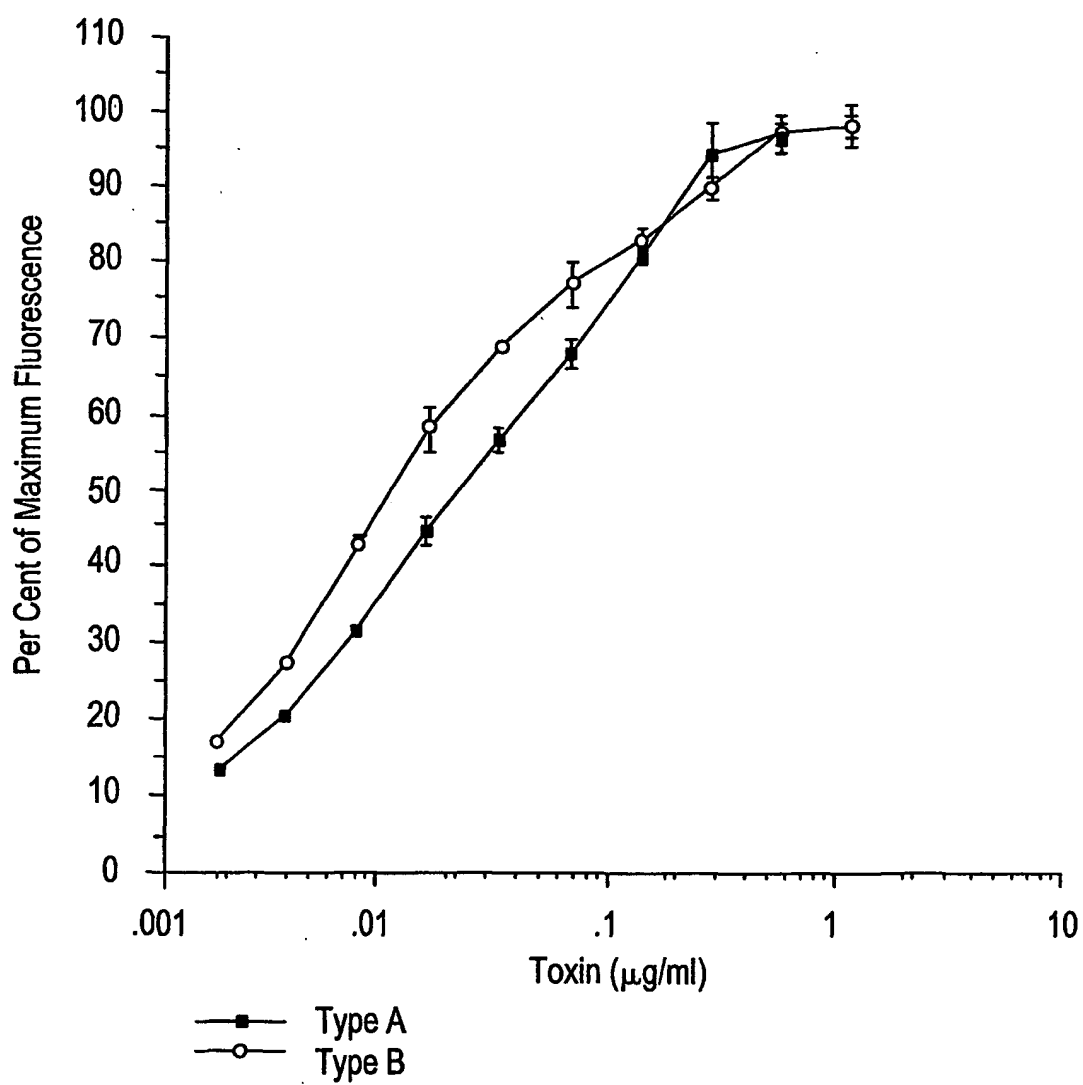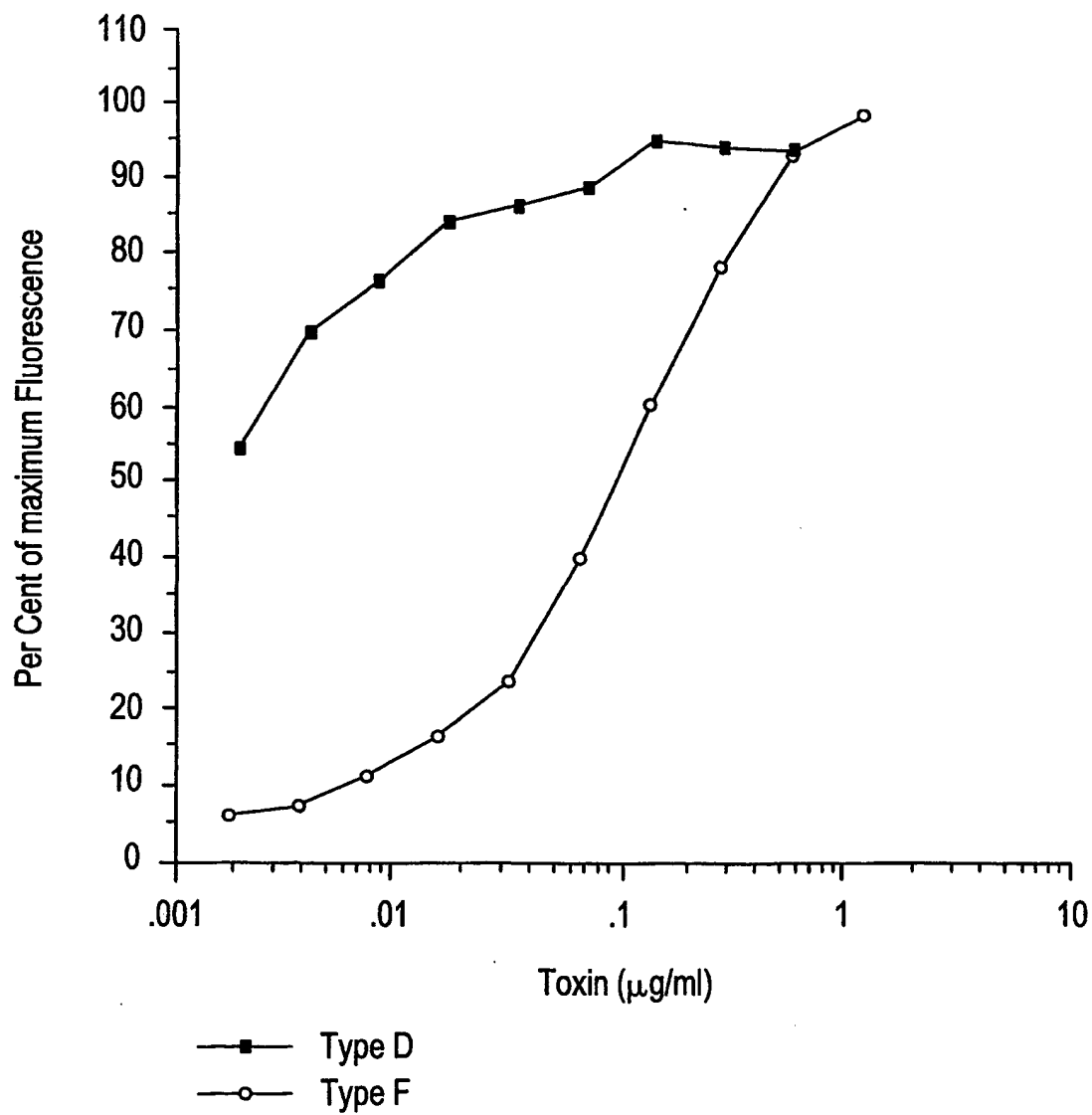
15

20

25

30

FIG. 1

FIG. 2a

FIG. 2b

Sequence Listing

1. SEQ ID NO:1

S   N   R   T   R   I   D   X   A   N   Q   R   A   Z   R   M   L

    Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and
"Z" is S-(fluoresceinyl)-cysteine.

2. SEQ ID NO:2
S   N   R   T   R   I   D   E   A   N   X   R   A   dcC R   M   L

    Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and
"dcC" is S-(7-dimethylamino-4-methyl-coumarin-3-
carboxamidomethyl)-cysteine.

SEQ ID NO:3
L   S   E   L   D   D   R   A   D   A   L   Q   A   X   A   S   Q   F   E   Z
S   A   A   K   L   K   R   K   Y   W   W   K   N   L   K

    Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and
"Z" is S-(fluoresceinyl)-cysteine.

SEQ ID NO:4
L   S   E   L   D   D   R   A   D   A   L   Q   A   G   A   S   X   F   E   dcC
S   A   A   K   L   K   R   K   Y   W   W   K   N   L   K

    Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and
"dcC" is S-(7-dimethylamino-4-methyl-coumarin-3-
carboxamidomethyl)-cysteine.

SEQ ID NO:5
A   Q   V   D   E   V   V   D   I   M   R   V   N   V   D   K   V   L   X   R
K   L   Z   E   L   D   D   R   A   D   A   L   Q   A   G   A
S

    Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and
"Z" is S-(fluoresceinyl)-cysteine.

SEQ ID NO:6
A Q V D E V V D I M R V N V D K V L E R K

L dcC E L D D R A D A L Q A G A

S

    Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and
"dcC" is S-(7-dimethylamino-4-methyl-coumarin-3-
carboxamidomethyl)-cysteine.

SEQ ID NO:7
A Q V D E V V D I M R V N V D K V L E R K

L mcp E L D D R A D A L Q A G A S

    Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and
"mcp" is 2-amino-3-(7-methoxycoumarin-4-yl)-propionic acid.

SEQ ID NO:8
flG G G S N R T R I D E A N Q R A T R M L

G G G C

    Where flG is N-fluoresceinyl-glycine.

SEQ ID NO:9
flG G G L S E L D D R A D A L Q A G A S Q

F E T S A A K L K R K Y W W K N L K

G G G C

    Where flG is N-fluoresceinyl-glycine.

SEQ ID NO:10
flG G G A Q V D E V V D I M R V N V D K V

L E R D Q K L S E L D D R A D A L Q

A G A S G G G C

    Where flG is N-fluoresceinyl-glycine.

SEQ ID NO:11

Z N K L K S S D A Y K K A W G N N Q D G

V V A S Q P A R V V D E R E Q M A I

```
S  G  G  F  I  R  R  V  T  N  D  A  R  E  N  E  M  D
E  N  L  E  Q  V  S  G  I  I  G  N  L  R  H  M  A  L
D  M  G  N  E  I  D  T  Q  N  R  Q  I  D  R  I  M  E
K  A  D  S  N  K  T  R  I  D  E  A  N  Q  R
A  T  K  M  L  G  S  G  C
```

Where Z is S-fluoresceinyl-cysteine.

SEQ ID NO:12
```
Z  N  K  L  K  S  S  D  A  Y  K  K  A  W  G  N  N  Q  D  G
V  V  A  S  Q  P  A  R  V  V  D  E  R  E  Q  M  A
I  S  G  G  F  I  R  R  V  T  N  D  A  R  E  N  E
M  D  E  N  L  E  Q  V  S  G  I  I  G  N  L  R  H
M  A  L  D  M  G  N  E  I  D  T  Q  N  R  Q  I  D
R  I  M  E  K  A  D  S  N  K  T  R  I  D  E  A  N
Q  A  A  T  K  M  L  G  S  G  C
```

Where Z is S-fluoresceinyl-cysteine.